

SECTION I
AMENDMENTS TO THE SPECIFICATION

Please insert the following replacement paragraph at page 6, lines 19-22:

In another aspect the present invention provides for a fusion protein comprising a target protein linked to a domain, wherein the domain protein includes amino acid residues on the C-terminal comprising a variant of (E E D K L(F/Y) Q S(M/L/Y).SEQ ID NO: 7), wherein the C-terminal part of the domain causes an affinity for subtilisin or variants thereof.

Please insert the following replacement paragraph at page 20, line 30 to page 21, line 4:

To demonstrate the feasibility of prodomain-directed processing, a gene was constructed to direct the synthesis of a fusion of the pR8 prodomain onto the N-terminus of the 56 amino acid B domain(GB) of streptococcal Protein G. Prodomain pR8 (SEQ ID NO: 4), having the mutations at amino acid residues 16-21 (QTMSTM (SEQ ID NO: 8)) which were replaced with SGIK creating a two amino acid deletion in pR8, wherein S replaces Q16, G replaces T17, M18I replaces S19 and T20 and “K” replaces M21; along with additional substitutions A23C, K27Q, V37L, Q40C, H72K and H75K is independently stable and binds to subtilisin with ~ 100-times higher affinity than the wild type prodomain. Further, pR8 thus becomes the cognate sequence specifying the subtilisin cleavage site.

Please insert the following replacement paragraph at page 21, lines 17-24:

Using pR8 to direct cleavage in and of itself does not create an optimal processing system because of subtilisin’s high activity against non-cognate sequences. The next step was to engineer subtilisin to be less active against non-cognate sequences. The starting point for engineering a processing subtilisin was a mutant denoted S149 : (Q2K, S3C,P5S, K43N, A73L, deletion of 75-83, E156S, G166S, G169A, S188P, Q206C, N212G, K217L, N218S, T254A and Q271E). S149 previously was engineered for high stability and ability to fold independently of the prodomain. These characteristics, while not essential, are highly desirable in a processing enzyme.

Please insert the following replacement paragraph at page 22, lines 1-11:

A version of pR8 was constructed with its last four amino acids (AHAY) replaced with FRAM (denoted pR58 (SEQ ID NO: 6)). pR58 inhibits S160 with aK_{1/2}of 30 pM. An N-terminal fusion of

pR58 onto the GB domain was found to bind to S160 with a substrate affinity (Ks) in the pM range, at least 4×10^5 -times greater than even the highly preferred pentapeptide substrate sDFRAM-AMC. Essentially the prodomain structure acts as an amplifier of the P1 and P4 sequence signals. Hydrolysis is limited to a single turn-over by strong product inhibition. Product inhibition is difficult to avoid in using high substrate affinity to direct specific cleavage because of the structural similarity between substrate and product. We therefore do not attempt to obviate this property. As will be described later, the single turn-over reaction can be exploited in applying the system to protein purification.

Please insert the following replacement paragraph at page 25, lines 4-9:

The prodomain of subtilisin can be replaced with a much shorter cognate sequence which has been selected for optimized binding with the processing protease. The amino acids comprising variations of only the C-terminal part of the prodomain (E E D K L(F/Y) Q S(M/L/Y).SEQ ID NO: 7) can be used as a cognate sequence. For example, it has been shown that the IgG binding domain of Streptococcal Protein G, which has no natural affinity to subtilisin, binds to S 194 with a sub-micromolar dissociation constant once a nine amino acid C-terminal tail has been added.

Please insert the following replacement paragraph at page 26, line 33 to page 27, line 2:

Further purification experiments were conducted on the 56 amino acid Streptococcal protein GB domain linked to pR58 (pR8FRAM.SEQ ID NO: 6) wherein the 671 fusion protein (pR58FKAM-GB SEQ ID NO: 5) was purified and separated on 189 HiTrap NHS column by continuous injection of 0. 1MI (F to demonstrate the effectiveness of the release of a target protein when mutant subtilisin was triggered by fluoride ions.

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